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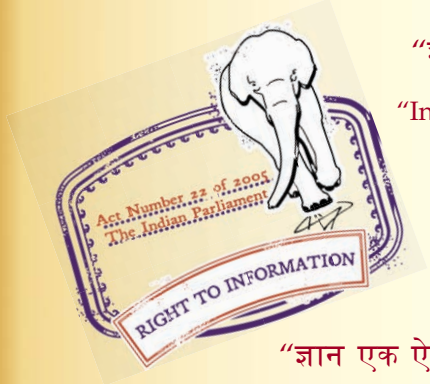
IS 5887-5 (1976): Methods for detection of bacteria responsible for food poisoning, Part 5: Isolation, identification and enumeration of VIBRIO CHOLERAE and VIBRIO PARAHAEMOLYTICUS [FAD 15: Food Hygiene, Safety Management and Other Systems]

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"Knowledge is such a treasure which cannot be stolen"

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IS : 5887 ( Part V ) - 1976
(Reaffirmed 2005)

*Indian Standard*

# METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

## PART V ISOLATION, IDENTIFICATION AND ENUMERATION OF *VIBRIO CHOLERAE* AND *VIBRIO PARAHAEMOLYTICUS*

*( First Revision )*

First Reprint MAY 1990

UDC 613.2-099 : 664 : 576.851.31.078



**BUREAU OF INDIAN STANDARDS**
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 1 iOO02

**Gr 4** *September* 1977

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## *( First Revision )*

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# *Indian Standard*

# METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

## PART V ISOLATION, IDENTIFICATION AND ENUMERATION OF *VIBRIO CHOLERAE* AND *VIBRIO PARAHAEMOLYTICUS*

## *( First Revision )*

## 0. FOREWORD

**0.1** This Indian Standard ( Part V ) ( First Revision ) was adopted by the Indian Standards Institution on 13 December 1976, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

**0.2** Several micro-organisms contaminating food give rise to clinical symptoms. These are abdominal pain, nausea, vomitting, diarrhoea and sometimes pyrexia. A well-known exception is that of botulism where the symptoms are those of difficulty in swallowing, diplopia, aphonia and difficulty in respiration. Poisoning through food is characterized by the explosive nature with which the symptoms occur in otherwise healthy individuals. Often several persons after having consumed a particular item of food, develop symptoms that serve as important guide in suspecting food poisoning. Such explosive nature of food poisoning helps in differentiating conditions from those of out-breaks of food-borne infectious diseases which generally spread over a period of several days. The micro-organisms causing food poisoning belong to bacteria, protozoa and helminths, fungi and viruses. However, this standard covers the methods for detection and estimation of important bacteria responsible for food poisoning and food-borne diseases.

**0.3** This standard was first published in 1970. It is being revised in parts covering methods of detection and estimation of various bacteria separately. This has been done with a view to making each part more comprehensive including various details of the method. It is expected that publication of these methods in parts will facilitate better implementation and adoption by the concerned organizations. This will also make

review and revision of the parts easier. The salient features of this revision are:

a) besides detection, estimation procedure for various organisms, where applicable, have been incorporated;

b) method of detection of *Vibrio parahaemolyticus* has been included; and

c) methods of identification have been updated.

**0.4** In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS:2-1960*.

## 1. SCOPE

**1.1** This standard ( Part V ) prescribes methods for isolation and identification of *Vibrio cholerae* and *Vibrio parahaemolyticus*, and their estimation in foods, if required to be carried out.

## 2. SAMPLING AND QUALITY OF REAGENTS

**2.1 Sampling** — For microbiological examination the sample should be handled carefully. For this purpose, IS:5404-1969† shall be followed.

**2.2 Quality of Reagents** — Unless specified otherwise, pure chemicals shall be employed in tests and distilled water ( *see* IS:1070-1960‡ ) shall be used where use of water as a reagent is intended.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

## 3. GENERAL CHARACTERISTICS OF *V. CHOLERAE* AND *V. PARAHAEMOLYTICUS*

**3.1** ***V. cholerae*** — The following are some of the characters of *V. cholerae*:

a) Gram negative rods,

b) Motile,

c) Catalase positive,

d) Oxidase positive,

e) Fermentative reaction in Hugh-Leifson's test,

*Rules for rounding off numerical values ( *revised* ).
†Code of practice for handling of food samples for microbiological analysis.
‡Specification for water, distilled quality ( *revised* ).

f) Ferments glucose without gas production,

g) Usually produces acid when fermenting *d*-mannitol,

h) Does not produce acid when fermenting *l*-inositol,

j) Does not produce $H_2S$ in TSI medium,

k) Usually grows in 1 percent tryptone broth,

m) Arginine dihydrolase negative,

n) Lysine decarboxylase positive, and

p) Ornithine decarboxylase positive.

The characters (a) to (j) are shown by strains of *Aeromonas* and *Plesiomonas*. These are differentiated from *Vibrio* in that *Aeromonas* strains are lysine decarboxylase negative, arginine dihydrolase positive; *Plesiomonas* strains do not ferment mannitol, usually ferment inositol and are lysine and ornithine decarboxylase positive and also arginine dihydrolase positive.

**3.2** ***V. parahaemolyticus***—The following are some of characters which have been recognized at present for the identification of *V. parahaemolyticus* strains:

a) Gram negative rod;

b) Oxidase positive;

c) Ferment glucose without gas when tested in Hugh-Leifson's test in the tube under paraffin seal;

d) Voges-Proskauer test negative;

e) Usually ferment mannitol;

f) Usually do not ferment sucrose;

g) Do not produce $H_2S$ in TSI medium;

h) Arginine dihydrolase negative;

j) Lysine decarboxylase positive;

k) Ornithine decarboxylase usually positive;

m) Growth at 42°C in tryptone broth, with added sodium chloride, positive;

n) Growth in 1 percent tryptone broth, with 8 percent sodium chloride, positive;

p) Growth in 1 percent tryptone broth, with 10 percent sodium chloride, negative ( an occasional strain may show growth ); and

q) Growth in 1 percent tryptone broth, without sodium chloride, negative.

## 4. MEDIUM

**4.1 Alkaline Peptone Water** — Dissolve 10 g peptone ( *see* IS:6853-1973* ) and 5 g sodium chloride in water and make up to 1 000 ml. Adjust *p*H to 8·2 and distribute into sterilized test tubes, and sterilize at 120°C for 15 minutes.

**4.2 Thiosulphate-Citrate-Bile Salts-Sucrose Agar ( TCBS )** — Dissolve by gentle heating 5 g yeast extract ( *see* IS:7004-1973† ), 10 g peptone ( *see* IS:6853-1973* ), 20 g sucrose, 10 g sodium thiosulphate ($NaS_2O_3,5H_2O$), 10 g sodium citrate ($Na_3C_6H_3,2H_2O$), 3 g sodium cholate, 5 g oxgall ( dehydrated bile ), 10 g sodium chloride, 1 g ferric citrate, 0·04 g bromothymol blue, 0·04 g thymol blue and 15 to 30 g agar ( *see* IS:6850-1973‡ ) in 1 000 ml of water. Boil for 1 to 2 minutes only. This medium shall not be further sterilized by autoclaving. The final *p*H of the medium shall be 8·6. Melt and pour into sterile petri dishes and allow to cool.

NOTE 1 — Sodium cholate can be omitted when 8 g of oxgall is to be used.

NOTE 2 — It is recommended that if this medium is being prepared as given above, the prepared medium be tested in parallel with a dehydrated commercially prepared TCBS medium to be confident that the medium prepared from the ingredients is satisfactory.

**4.3 Bile Salt Agar** — Dissolve by steaming in 1 000 ml of water, 10 g peptone ( *see* IS:6853-1973* ), 5 g meat extract ( *see* IS:6851-1973§ ), 5 g sodium chloride and 5 g sodium taurocholate. Add 15 to 30 g agar ( *see* IS:6850-1973‡ ), mix and dissolve by heating. Adjust *p*H to 8·5 with sodium hydroxide solution. Cool and filter through a filter paper ( Whatman No. 1 or equivalent ) or absorbent cotton wool previously wetted with water. Distribute into sterile flasks in convenient amounts and sterilize by autoclaving at 120°C for 15 minutes. Plates are made by melting the stock medium and pouring into sterile petri dishes.

**4.4 Nutrient Agar** — Mix and dissolve by heating 10 g peptone ( *see* IS:6853-1973* ), 10 g meat extract ( *see* IS:6851-1973§ ) and 5 g sodium chloride in 1 000 ml water. When cool, adjust *p*H to 7·5 to 7·6. Remove precipitate by filtering through filter paper. Add, and dissolve by heating, agar ( *see* IS:6850-1973‡ ) in such a concentration as will solidify and produce a sufficiently firm surface when poured in sterile petri dishes or made as slopes. The concentration of agar to be added varies from batch to batch of agar, and needs to be adjusted accordingly. Usual concentrations required vary from 1·5 to 3 percent. Sterilize by autoclaving at 120°C for 15 minutes. Plates and slopes are prepared from sterilized nutrient agar.

*Specification for peptone, microbiological grade.
†Specification for yeast extract, microbiological grade.
‡Specification for agar, microbiological grade.
§Specification for meat extract, microbiological grade.

**4.4.1** *Nutrient Agar for the Motility Test* — The medium consists of 3 g meat extract ( *see* IS:6851-1973* ), 10 g peptone ( *see* IS:6853-1973† ), 5 g sodium chloride, and 4 to 5 g agar ( *see* IS:6850-1973‡ ) dissolved in 1000 ml of water. Adjust *p*H to 7·5 to 7·6. Put the dissolved medium into test tubes to fill a part of the tube and place into this a glass tube open at both ends. One end of the glass tube shall project from above the surface of the agar for not less than 1·5 cm. Sterilize the tubes with the medium at 120°C for 15 minutes, and then cool. The consistency of the agar should be soft but not liquid. This is achieved by altering the amount of agar used, if necessary.

**4.5 Medium for Hugh-Leifson's Test** — Dissolve by heating in 1000 ml water, 2 g peptone ( *see* IS:6853-1973† ), 5 g sodium chloride, 0·3 g dipotassium hydrogen phosphate, and 3 g agar ( *see* IS:6850-1973‡ ). Adjust to *p*H 7·1, filter and add 15 ml 0·2 percent alcoholic solution of bromothymol blue. Sterilize at 115°C for 20 minutes. Add sterile solution of glucose to give a final concentration of 1 percent. Mix and distribute in tubes, 14 × 100 mm, adding 3 to 4 ml per tube.

NOTE — In carrying out the test with *V. parahaemolyticus*, the medium as in **4.5** should contain an additional 2 to 3 percent sodium chloride.

**4.6 Peptone Water Medium for Carbohydrate Fermentation Tests** — Dissolve 10 g peptone ( *see* IS:6853-1973† ) and 5 g sodium chloride in 900 ml of water and adjust the *p*H to 7·1 to 7·3 so that after addition of 10 ml of Andrade's indicator the *p*H shall be 7·5. Sterilize at 115°C for 20 minutes. Dissolve 10 g of the requisite sugar in 90 ml of water and steam for 30 minutes or sterilize by filtration. With sterile precautions, add this 90 ml of sugar solution and 10 ml of Andrade's indicator solution ( prepared by adding 1 N sodium hydroxide solution to 0·5 percent aqueous solution of acid fuchsin until the colour of the indicator solution is just yellow ) to 900 ml of the sterile peptone water solution. Distribute into sterile test tubes containing inverted Durham's fermentation tubes, and steam for 30 minutes.

NOTE — For carrying out the fermentation tests with *V. parahaemolyticus*, the medium as in **4.6** should contain an additional 2 to 3 percent sodium chloride.

**4.7 TSI Medium for $H_2S$ Test** — Heat to dissolve in 1000 ml water, 3 g meat extract ( *see* IS:6851-1973* ), 3 g yeast extract ( IS:7004-1973§ ), 20 g peptone ( *see* IS:6853-1973† ), 1 g glucose, 10 g lactose, 10 g sucrose, 0·2 g ferrous sulphate ( $FeSO_4,7H_2O$ ), 5 g sodium chloride, 0·3 g sodium thiosulphate ( $Na_2S_2O_3,5H_2O$ ) and 15 to 30 g agar. Add 12 ml of

*Specification for meat extract, microbiological grade.
†Specification for peptone, microbiological grade.
‡Specification for agar, microbiological grade.
§Specification for yeast extract, microbiological grade.

0·2 percent phenol red solution, mix and tube. Sterilize by autoclaving at 115°C for 20 minutes. Pour into sterile test tubes and cool to form a slope with deep butts.

NOTE — For carrying the test with *V. parahaemolyticus*, the medium as in **4.7** shall contain an additional 2 to 3 percent sodium chloride.

**4.8 Tryptone Broth Medium** — Tryptone broth medium, without added sodium chloride, is prepared by dissolving 10 g tryptone ( *see* IS:7127-1973* ) in 1 000 ml water. Sodium chloride in requisite amount [ *see* **3.2** (m) to (p) ] is to be added where indicated, for example, for *V. parahaemolyticus*. The medium is dispensed in 5 ml amounts into sterilized tubes and autoclaved at 120°C for 15 minutes.

**4.9 Medium for Voges-Proskauer Reaction** — Steam to dissolve in 1 000 ml water, 5 g peptone ( *see* IS:6853-1973† ) and 5 g dipotassium hydrogen phosphate. Filter and adjust the *p*H to 7·5. Add 5 g glucose, mix to dissolve; distribute into tubes. Sterilize at 115°C for 10 minutes.

NOTE — For carrying out the test with *V. parahaemolyticus*, the medium as in **4.9** shall contain an additional 2 to 3 percent sodium chloride.

**4.10 Medium for Dihydrolase and Decarboxylase Activity** — Dissolve with heat, 5 g peptone ( *see* IS:6853-1973† ) and 5 g meat extract ( *see* IS:6851-1973‡ ), 5 mg pyridoxal, 0·5 g glucose in 1 000 ml water. Adjust the *p*H to 6·0 and add 5 ml of 0·2 percent solution of bromocresol purple and 2·5 ml of 0·2 percent solution of cresol red. Sterilize in an autoclave at 115°C for 20 minutes. Divide this base medium into four equal volumes, and to each of three add the respective amino acid ( arginine, lysine and ornithine ) as a final concentration of 1 percent if the *l*-form of the amino acid is used or as 2 percent if the *dl*-form of the amino acid is used. The fourth aliquot of the base medium does not contain amino acid and serves as control. Distribute the four media in 1- to 1·5-ml amounts into small sterilized tubes, 67 × 10 mm, and layer with sterile liquid paraffin to a height of about 5 mm. The *p*H of the final medium is to be readjusted, if necessary, to 6·0. Sterilize in an autoclave at 115°C for 10 minutes.

**4.10.1** An alternative medium where the base medium contains 5 g peptone ( *see* IS:6853-1973† ), 3 g yeast extract ( *see* IS : 7004-1973§ ), 1 g glucose and 10 ml of 0·2 percent bromocresol purple in 1 000 ml of water may be used. Adjust the *p*H to 6·7. The remaining procedure for preparation of the medium is as in **4.10**.

NOTE — For carrying out the tests with *V. parahaemolyticus*, the media as in **4.10** and **4.10.1** shall contain an additional 2 to 3 percent sodium chloride.

*Specification for tryptone, microbiological grade.
†Specification for peptone, microbiological grade.
‡Specification for meat extract, microbiological grade.
§Specification for yeast extract, microbiological grade.

**4.11 Glucose-Salt-Teepol Broth** — Dissolve in 1 000 ml water, 3 g meat extract ( *see* IS:6851-1973* ), 10 g peptone ( *see* IS:6853-1973† ), 30 g sodium chloride, 5 g glucose, 0·002 g methyl violet and 4 ml Teepol. This gives single-strength broth. When double-strength broth is required, the ingredients in double the amount are dissolved in 1 000 ml water. Sterilize by autoclaving at 120°C for 15 minutes. The final *p*H should be 7·4.

**4.12 Media for the Kanagawa Phenomenon for *V. parahaemolyticus***

**4.12.1** *Trypticase-Soy-Sodium Chloride Broth* — Dissolve by boiling for 1 to 2 minutes 15 g trypticase or tryptone ( *see* IS:7127-1973‡ ), 5 g phytone ( or soytone ), 30 g sodium chloride, 2·5 g dipotassium phosphate, 2·5 g glucose in 1 000 ml water. Dispense into sterilized test tubes, and autoclave at 120°C for 15 minutes. The final *p*H should be 7·3.

**4.12.2** *Wagatsuma Blood Agar* — Dissolve by gentle heating in 1 000 ml water, 3 g yeast extract ( *see* IS:7004-1973§ ), 10 g peptone ( *see* IS:6853-1973† ), 70 g sodium chloride, 5 g dipotassium phosphate, 10 g mannitol, 0·000 1 g crystal violet, and 15 to 30 g agar ( *see* IS:6850-1973‖ ). Adjust *p*H to 8·0. Do not autoclave but steam for 30 minutes and cool to about 50°C. Add 100 ml of a 20-percent suspension of freshly drawn citrated human red cells which have been washed three times in sterilized physiological saline. Mix well and pour on to sterilized petri dishes and cool. The plates shall be used as soon as is possible and should be thoroughly dry before use.

## 5. PROCEDURE FOR ISOLATION

**5.1** ***Vibrio cholerae*** — Where necessary, the sample may be blended in a sterile blender jar for 2 minutes or macerated with sterile sand in a sterile mortar using approximately 200 ml of alkaline peptone water ( *see* **8.1** ) per approximately 25 g of the sample. The specimen is inoculated in each of the three media ( **4.1**, **4.2** and **4.3** ) and incubated at 37°C overnight. Subcultures are made from the liquid medium on each of the two solid media ( **4.2** and **4.3** ) for further overnight incubation. The suspicious colonies of Vibrios in TCBS medium ( **4.2** ) appear yellow coloured with entire round margins. On bile salt agar ( **4.3** ), colonies of Vibrios have a distinctive appearance which may be seen by growing a known strain of *V. cholerae* on this medium and comparing it with, say, that of *Esch. coli*. As a preliminary test only, suspicious growths may be tested by slide agglutination using polyvalent cholera typing serum

*Specification for meat extract, microbiological grade.
†Specification for peptone, microbiological grade.
‡Specification for tryptone, microbiological grade.
§Specification for yeast extract, microbiological grade.
‖Specification for agar, microbiological grade.

( high titre serum of combined Ogawa and Inaba serotypes ). The growth is emulsified in a drop of normal saline and smooth suspensions mixed with a drop of cholera serum. Positive reaction is shown by the appearance of clumps within 30 seconds. When testing directly from the two solid media mentioned, only a portion of the colony should be used for the preliminary slide agglutination test, and the remaining portion of the positive colony is replated on another bile salt agar plate ( **4.3** ) to obtain larger yield of pure colonies. Colonies of Vibrios which agglutinate with polyvalent cholera typing serum belong to *V. cholerae* serotype 1 considered to be the principal cause of cholera in man. It should however be noted that there are other serotypes of *V. cholerae*, and that strains in the R ( rough ) form cannot be serotyped.

NOTE — In case TCBS medium ( **4.2** ) is not available to the laboratory, inoculation on this medium may be omitted.

**5.1.1** It is essential that all suspect Vibrio colonies, whether positive or negative by preliminary slide agglutination test, are tested and identified as *V. cholerae* when exhibiting the characters as in **3.1**.

**5.2** ***Vibrio parahaemolyticus*** — The specimen, if necessary, homogenized as in **5.1** is inoculated in glucose-salt-Teepol broth ( **4.11** ) and incubated overnight at 37°C. Inoculum from the growth is streaked on to TCBS medium ( **4.2** ) and incubated for 18 hours at 37°C. The colonies of *V. parahaemolyticus* on TCBS medium are round, about 2 to 3 mm in diameter, and are with green or blue centres. Such colonies are to be identified as *V. parahaemolyticus*, when exhibiting the characters as in **3.2**.

## 6. TESTS FOR IDENTIFICATION

**6.1 Gram's Stain** — The stain consists of (a) 0·5 percent methyl violet or crystal violet in water, (b) iodine solution ( 1 percent iodine and 2 percent potassium iodide in water ), and (c) counterstain ( 0·1 g neutral red, 0·2 ml of 1·0 percent acetic acid and 100 ml water ).

On a clean grease-free slide, very light and thin smear covering a small area is made directly from liquid culture and in clean tap water if from solid media. The smear is fixed by passing to and fro over a flame and cooled. Cover the smear with the stain (a) for 30 seconds, pour off the stain and wash with (b) and than cover with (b) and allow to remain for 30 seconds. Wash off with ethanol until the dye ceases to stream out. Wash in running tap water and apply (c) for about one minute. Wash in tap water and dry for examination.

**6.2 Test for Motility** — Inoculate by stabbing with a straight wire into the top of the medium as in **4.4.1**, the strain to be tested inside the glass tube to a depth of about 5 mm. Take care that inoculation is

not made on to the surface of the medium outside the glass tube. Incubate at 37°C for 18 to 24 hours. Motile strains shall be found to show growth on the surface of the medium outside the 'inner glass tube' having travelled through the entire medium inside this inner tube. If negative on the first day, keep the inoculated tube at room temperature for a further 4 to 6 days to see if evidence of motility is present.

**6.3 Test for Catalase** — Grow strain for 18 to 24 hours at 37°C on nutrient agar ( **4.4** ) slope and pour 1 ml of hydrogen peroxide over the growth with the tube in a slanting position. Release of oxygen, shown as bubbles, from hydrogen peroxide indicates presence of catalase.

**6.4 Test for Oxidase** — To nutrient agar ( **4.4** ) slope of the culture, freshly grown, is added a few drops of freshly mixed test reagents, that is, 1 percent solution of alpha-naphthol in 95 percent ethanol and equal amount of 1 percent solution of *p*-aminodimethylaniline hydrochloride in water. A positive reaction is indicated by the appearance of a blue colour within two minutes.

NOTE — For carrying out the test with *V. parahaemolyticus*, nutrient agar as in **4.4** shall contain an additional 2 to 3 percent sodium chloride.

**6.5 Hugh-Leifson's Test** — The strain from fresh nutrient agar ( **4.4** ) growth is stabbed into two tubes of medium ( **4.5** ), one of which is then layered over with a small amount of sterile liquid paraffin. Incubate both tubes at 37°C and observe up to 4 days. Acid formation, shown by yellow colour, in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction. Lack of acid in either tube indicates the strain as not being able to utilize glucose oxidatively or fermentatively.

NOTE — For *V. parahaemolyticus*, the medium given in **4.5** should contain additional sodium chloride.

**6.6 Tests for Fermentation of Carbohydrates**

**6.6.1** *V. cholerae* — Inoculate each of peptone water medium ( **4.6** ) containing respectively, mannitol, inositol and glucose and incubate at 37°C for 18 to 48 hours.

**6.6.2** *V. parahaemolyticus* — Inoculate each of peptone water medium with added sodium chloride containing respectively, mannitol and sucrose. Incubate at 37°C for 4 to 5 days.

**6.7 Test for $H_2S$ Production** — Inoculate TSI medium ( **4.7** ) by stabbing the strain into the butt and streaking the slope. Incubate at 37°C and observe daily for up to 7 days. The presence or absence of blackening in the butt of the medium is to be recorded.

NOTE — For *V. parahaemolyticus*, the medium given in **4.7** should contain additional sodium chloride.

**6.8 Test for Growth in Tryptone Broth**

**6.8.1** *V. cholerae* — Inoculate the medium as in **4.8** without addition of sodium chloride. Incubate for 18 hours at 37°C.

**6.8.2** *V. parahaemolyticus* — Inoculate medium as in **4.8**, with and without added sodium chloride as required for eliciting the characters as in **3.2** (m) to (q). Incubate for 24 hours, at 37°C for characters (n) to (q) and at 42°C for character (m).

**6.9 Test for Voges-Proskauer Reaction (for *V. parahaemolyticus*)** — Inoculate the medium ( **4.9** ) with added sodium chloride and incubate at 37°C for 48 hours. To 1 ml of the growth add 0·6 ml of 5 percent alpha-naphthol prepared in ethanol. Shake and add 0·2 ml of 40 percent aqueous potassium hydroxide solution. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicate a positive rection.

**6.10 Test for Dihydrolase and Decarboxylase Activities** — Inoculate, using a straight wire, through the liquid paraffin each of the four tubes of the medium as described in **4.10** from a freshly grown culture in nutrient agar ( **4.4** ). Incubate at 37°C and examine daily for up to 4 days only. The medium first becomes yellow due to acid production from the glucose, later, if dihydrolation or decarboxylation of the respective amino acid occurs, the medium becomes violet in colour.

NOTE — For *V. parahaemolyticus*, the medium ( **4.10** ) should contain additional sodium chloride.

When performing the test, always use as control a known strain of *V. cholerae*, and a known strain of *V. parahaemolyticus*, obtained from a reference laboratory.

**6.11 Additional Test Which May be Applied to *V. parahaemolyticus* to Suggest Pathogenicity of the Strains (Kanagawa Phenomenon)** — A positive Kanagawa phenomenon has been found to correlate closely with the pathogenicity of strains of *V. parahaemolyticus*, and is useful when isolated from human sources. It is, however, mentioned that isolates recovered from sea-foods are almost always Kanagawa negative.

The Kanagawa test is carried out by spotting a loopful of an 18 hour culture grown in broth as in **4.12.1** on to blood agar plates as in **4.12.2**, and incubating at 37°C for *not more than* 24 hours. A positive result consists of a zone of transparent clearing of the red blood cells around the colony. It is very important that the reading is not taken beyond 24 hours of incubation as any haemolyses seen beyond this time is not to be recorded as Kanagawa positive.

## 7. SEROTYPING AND PHAGE TYPING

**7.1** ***V. cholerae*** — Serotyping and phage typing of the strains are best made at laboratories especially undertaking such work.

NOTE — It is also brought to notice that in the Report of the Subcommittee on Taxonomy of Vibrios to the International Committee on Nomenclature of Bacteria, it has been pointed out that *V. cholerae* includes strains which may or may not elicit nitroso indole reaction, may or may not be haemolytic, may or may not be sensitive to polymyxin B, may or may not be agglutinated by Gardner and Venkataraman O group antiserum, may or may not be lysed by Mukherjee *V. cholerae* bacteriophage I, II, III, IV and V (International Journal of Systematic Bacteriology, *22*, p 123, 1972).

**7.2** ***V. parahaemolyticus*** — Serological typing of *V. parahaemolyticus* can be carried out at present only in a very few selected laboratories in the world.

## 8. ENUMERATION

**8.1 Pre-enrichment and Blending** — Take 50 g of the sample in a sterile blender jar and to this add 450 ml of a sterile solution containing 0·1 percent peptone ( *see* IS:6853-1973* ) and 0·8 percent sodium chloride at *p*H 7·8 to 8·0 for enumerating *V. cholerae*. For *V. parahaemolyticus*, 450 ml of sterile 3 percent sodium chloride solution only is added. Keep for about 15 minutes at room temperature. Blend at 8000 to 10000 rev/min for 2 minutes. If the temperature of the contents of the mixture increases by more than 5°C, the procedure of blending is to be carried out by keeping the container in an ice-bath. After blending, allow the solid particles, if any, to settle down. If a blender is not available, macerate in a sterile mortar with sterile sand.

**8.2 Dilutions** — The procedure as in **8.1** provides a dilution of $10^{-1}$ and is then serially diluted ten-fold with the same solutions as in **8.1**, for *V. cholerae* and *V. parahaemolyticus*.

**8.3 Enumeration**

**8.3.1** *V. cholerae* — Ten-fold dilutions of the sample up to $10^{-4}$ as in **8.2**, are taken and from each dilution and 10 ml aliquots are inoculated into 10 ml of medium consisting of 2 percent peptone ( *see* IS:6853-1973* ) and 2 percent sodium chloride at *p*H 7·8 to 8·0. Incubate at 37°C for 18 hours, and streak a measured loopful (3 mm) from each tube on to bile salt agar medium as in **4.3**. Incubate at 37°C for 18 hours. Count the colonies of *V. cholerae*, whose characters are as described in **5.1**. Confirm the suspect colonies as being *V. cholerae* by the characters as in **3.1** and by the tests which have been described in **6**. Calculate the number of viable colonies of *V. cholerae* per gram of sample by multiplying by the dilution factor(s) and dividing by the mass of the sample.

*Specification for peptone, microbiological grades.

**8.3.2** *V. parahaemolyticus* — Ten-fold dilution of the sample up to $10^{-4}$, as in **8.2**, are taken, and from each dilution 10 ml aliquots are inoculated into 10 ml of double-strength glucose-salt-Teepol broth ( **4.11** ) and incubated overnight at 37°C. Measured loopful ( 3 mm ) from each tube is streaked on to TCBS medium ( **4.2** ) and incubated for 18 hours at 37°C. The characteristic colonies of *V. parahaemolyticus* as described in **5.2** are counted. These colonies are to be confirmed as being *V. parahaemolyticus* by the characters as in **3.2** and by the tests which have been described in **6**. Calculate the number of viable colonies of *V. parahaemolyticus* per gram of sample by multiplying by the dilution factor(s) and dividing by the mass of the sample.

NOTE — Alternative method of enumeration is to prepare ten-fold dilutions of the sample upto $10^{-4}$ as in **8.2** and to inoculate 0·1 ml amounts on to the surface of bile salts agar medium (**4.3**) for *V. cholerae* and on to TCBS medium (**4.2**) for *V. parahaemolyticus*. The inoculum is streaked evenly on to well-dried surface of the respective medium. Incubate at 37°C for 18 hours, and then proceed as in **8.3.1** for *V. cholerae* and **8.3.2** for *V. parahaemolyticus*.

( *Continued from page* 2 )

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